Polymorphic Genetic Markers in Amniotic Fluid

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A. INTRODUCTION

A.1 Objective

We propose to study amniotic fluid (AF) and cultured amniotic fluid cells for the expression and variation in the expression of various polymorphic genes. As the loci of those polymorphic genetic markers which are expressed in AF are shown to be closely linked to loci of genes determining disease, we will use the specific syntenic relationship for prenatal detection of the disease in the fetus.

A.2 BACKGROUND AND RATIONALE

Surprisingly little is known about the origin of AF, and it appears that various sources contribute to the 98% water and 2% solids making up this biological fluid of the fetal environment (1). Fetal urine, amniotic epithelium, cells of the fetal respiratory tract and, perhaps, the umbilical cord are thought to contribute at different times during gestation (2). The fetal cells suspended in the AF probably represent exfoliation from the skin, umbilical cord, urinary tract, oropharyngeal mucosa and amnion (3). Erythrocytes which are seen following the majority of amniocentesis procedures are almost certainly maternal in origin. The soluble and cellular constituents of AF provide the opportunity to study a sample of the genome of the unborn fetus, especially genetic systems of antigens and enzymes. Detection of certain of the latter in cultured AF cells, of course, forms the basis for prenatal diagnosis of some of the hereditary metabolic errors (4). It is possible to study some polymorphic genetic systems in the fetus from AF analysis (5,6,7), although a systematic search for expression of human polymorphisms in AF has not been undertaken. The emphasis on prenatal diagnosis of hereditary disorders has, understandably, focussed on detection in AF of enzymes whose deficiencies cause disease, and these genetic markers are usually idiomorphs (8). The variation among individuals implied by polymorphic genetic systems adds a dimension to the research design which allows certain areas of genetic significance to be explored. Comparision of expressions in the fetus of differing alleles at the same locus can be made. The investigator can pursue the effects of feto-maternal incompatibility on expressions of various alleles.

It is clear that a significant scientific advance has been encountered in the successful exploitation of amniotic fluid for the prenatal diagnosis of genetic disorders. The ability to isolate cells of fetal origin from AF and successfully culture them is the basis for monitoring "high risk" preganancies to detect fetuses with various chromosomal aberrations and some

inborn errors of metabolism (4). The technique of prenatal diagnosis removes the uncomfortable uncertainty from genetic counseling and, coupled with selective abortion of affected fetuses, joins the armamentarium of preventive medicine. As with most desirable techniques, there are limitations. Considerable time may elapse between amniocentesis and diagnosis in order to obtain sufficient numbers of cultured AF cells required for a particular test, usually biochemical in nature. Sufficient cells must be available for testing by (approximately) 20 weeks of gestation, i.e. the deadline for performing a therapeutic abortion. With reference to this limitation, the development of methodology which accurately tests the AF, or AF cells, at once is desirable. Another and more fundamental limitation presently confronting prenatal diagnostic activities arises from the inability to detect phenotypes at the cellular level. For some diseases, although the biochemical phenotype is known, absence of the normal protein from AF cells prevents their detection in the fetus; sickle cell anemia, hemophilia, phenylketonuria and ornithine transcarbamylase are some of these prenatal diagnostic "orphans". For other hereditary diseases, sufficient information on the underlying biochemical defect is not available, and numerous autosomal recessive, autosomal dominant and X-linked pathologic characters can be cited for this category. Cystic fibrosis (although this disorder may soon be liberated from this category), X-linked ichthyosis, neurofibromatosis and Huntington's Chorea are just a few. While we can optimistically look forward to increasing progress in the elucidation of basic mechanisms of these hereditary disorders, a waiting period before application to prenatal diagnosis of these conditions is not necessarily implied. In other words, even though a biochemical or other suitable cellular phenotype may not be available, it may still be possible to diagnose with considerable accuracy some of these conditions in the fetus. Genetic linkage may offer this possibility.

Human linkage (syntenic) groups are relevant to prenatal diagnosis of inherited disorders because the detection of the phenotype of a genetic marker which is closely linked to the locus of an allele determining disease provides the possibility of genotyping the fetus. This is already possible for myotonic dystrophy, a disease expressed in heterozygotes for an allele at a locus which is linked to the ABH secretor locus, the recombination fraction being 0.04 (9). ABH substances are secreted early in gestation by the fetus into amniotic fluid, forming the basis of determining its secretor status (5). Thus, if the coupling phase is known for an individual who is heterozygous at both loci and married to a non-secretor, detection of the secretor status of the fetus will predict presence or absence of the allele determining myotonic dystrophy. The magnitude of error in this prediction, 8%, is slightly greater than the recombination fraction (9).

The genetic map of man is growing. More and more linked loci are being found, autosomal linkage groups containing more than two loci are recognized and loci are being assigned to visible

autosomal chromosomes (10). The majority of the newer linkage groups involve polymorphic biochemical or serological markers rather than inherited disease markers. This is understandable because ascertainment of infrequent diseases in families is by-passed and accumulation of data is facilitated. Furthermore, linkage analysis by in vitro, Sendai virus mediated, interspecific somatic cell hybridization (11) does not require polymorphic gene markers so long as differences between interspecific allozymes or other homologous markers can be detected. Still, for prenatal diagnosis, synteny involving a locus with a disease determining allele is essential.

At present, a small number of autosomal syntenic groups involving clinically significant loci are known. The loci of five autosomal dominant disorders have been shown by family studies to be linked to polymorphic loci (reviewed in reference 10):

MAIN LOCUS	TEST LOCUS	DISTANCE CENTIMORGANS	95% PROBABILITY LIMITS OF MAP DISTANCE CENTIMORGANS	COMMENTS
Congenital total nuclear cataract	Duffy	0	0-17	Probably syntenic with the two loci for pancreatic amylase. Assigned to chromosome #1.
Elliptocytosis	Rh	3	2-7	Syntenic with loci for 6-phosphogluconatedehydrogenase & phosphoglucomutase (first locus), and peptidase C. If these loci are correctly assigned to chromosome #1, they are syntenic with loci for the Duffy system, congenital total nuclear cataract and pancreatic amylase. Linkage studies with Rh have detected genetic heterogeneity for elliptocytosis.
Nail-Patella	ABO	13	8-21	
	Adenylate Kinase	0	0-6	
Sclerotylosis	MNS	4	0.3-19	
Myotonic Dystrophy	Secretor	4		

The biochemical basis of almost all autosomal dominant disorders, including those listed above, is unknown, denying us a rational foundation for prenatal diagnostic testing. Genetic linkage affords a potentially useful strategy for antenatal detection of autosomal dominant disorders. This also applies at present to some clinically significant loci on the human X chromosome:

MAIN LOCUS	TEST LOCUS	DISTANCE CENTIMORGANS	95% PROBABILITY LIMIT OF MAP DISTANCE CENTIMORGANS	COMMENTS
Hemophilia A (Factor VIII deficiency)	Glucose-6-phosphate dehydrogenase	4	0-16	Polymorphism at the test locus in black, Mediterranean & some Asiatic populations
Ichthyosis	Xg	18	11-31(*)	
Ocular Albinism	Xg	18	7-34(*)	

(references 12,13 and 14, respectively)
(*) - 90% probability limits

With respect to the above autosomal syntenic groups it should be pointed out that the ABO types of secretor + fetuses and secretor types have been detected from AF (5), and that 6-phosphogluconate dehydrogenase (6-PGD), of the Rh syntenic group, has been detected in cultured AF cells (7). The electrophoretic phenotype of glucose-6-phosphate dehydrogenase (G-6-PD) has also been detected in cultivated AF cells (7). Thus, the potential exists for diagnosing the nail patella syndrome, myotonic dystrophy, hemophilia A and perhaps elliptocytosis in fetuses at risk by virtue of syntenic relationships.

It is clear that, at present, there are limitations in the use of syntenic groups for prenatal diagnosis of inherited disorders. The method will be useful only for informative families, i.e. those with a doubly heterozygous (at test and main loci) parent for autosomal dominant traits, a doubly heterozygous mother for X-linked recessive characters and both parents doubly heterozygous for autosomal recessive disorders. Meiotic crossing over between the marker (test) and main loci will always provide the possibility of error in predicting the fetal genotype, and the magnitude of this error is provided by the recombination frequency. The smaller the distance between these loci, the lower this error will be. Still this error will be, in general, much less than the 50% "error" that now attends genetic counseling based on detecting a male fetus at risk for an X-linked disorder.

Another limitation of utilizing linkage information for prenatal diagnosis is the small number of syntenic groups involving clinically significant loci. At present, there is good reason to be optimistic about mapping the human chromosomes. Ruddle has indicated that each of the chromosomes will have a known linkage assignment within "the next several years" (15). Family studies and interspecific somatic cell hybridization are providing data for new syntenic groups, assignment to recognized groups and to visible chromosomes, and these in vivo and in vitro techniques for linkage analysis are also confirming each other's findings (e.g. reference 16). We should inject a slightly less optimistic comment concerning the application of genetic linkage groups detected by somatic cell hybridization to prenatal diagnosis of inherited disorders. The genetic markers used in these studies with interspecific hybrids are usually enzymes which are not necessarily polymorphic characters, as classified by electrophoresis. This may be especially true for autosomal recessive genes determining various metabolic errors. For prenatal detection of autosomal recessive disorders, both carrier parents must also be heterozygous, preferably for codominant alleles, at the linked test locus, an unlikely expectation if the test locus is not polymorphic. Thus, this restricts the number of marker loci and the frequency of couples at risk which are informative for application of linkage for the prenatal diagnostic test. The situation is only slightly improved for X-linked loci because of the paucity of X-linked polymorphic systems (Xg, Xm and in some populations, glucose-6-phosphate dehydrogenase; color blindness is a recessive trait).

Another problem with linkage data collected from analysis of somatic cell hybrids resides in the inability to estimate the frequency of recombination between test and main loci. For purposes of prenatal detection, the recombination frequency is an estimate of the error in the prediction of the fetal genotype and phenotype. The limitation in observing polymorphism at a number of the (enzyme) loci which will be linked by this technique suggests that for some linkages, confirming family studies will not be forthcoming because informative families will be rarely encountered.

Despite these reservations, the more linkages determined between loci and between loci and identifiable chromosomes, the more likely this knowledge can be applied to prenatal diagnosis of various inherited disorders. Certainly we can expect assignment of polymorphic loci to linkage groups and visible chromosomes, and detection of polymorphism in a number of presently monomorphic or idiomorphic systems is more than a reasonable prediction. The amount of genetic variability in terms of polymorphism, has surpassed estimates of a mere decade ago, and, indeed, it is not unreasonable to consider the possibility that almost all loci are polymorphic in man (17). Thus, we can expect assignment of a number of clinically significant loci to syntenic groups.

A final limitation to the use of synteny for prenatal diagnosis stems from the relatively small amount of information available on the expression of polymorphic genetic markers in AF. Should additional, clinically relevant, syntenic groups be detected, we still must be able to test for the expression of the marker loci in AF in order fo infer the fetal genotypes. In addition to providing information pertaining to the biology of the fetus and AF, a systematic study of polymorphisms in AF can provide the information necessary to use syntenic relationships for the prenatal diagnosis of inherited disorders.

B. SPECIFIC AIMS

1. We propose to seek in AF and cultured AF cells the expression of known polymorphisms. Among the polymorphisms to be studied will be erythrocyte antigenic systems, the HL-A system and systems of various intracellular enzymes.

2. We will delineate variation in expression of allelic markers in AF and attempt to understand the basis of the variation.

3. We will delineate expression of polymorphic markers in AF in terms of gestational age.

4. We will compare the expression of polymorphic markers in AF with their expression in other tissues (e.g. blood, cultured fibroblasts, etc.).

5. As we come to understand the variation encountered in the expression of polymorphic markers in AF and the limitations of the tests we use, we shall begin to employ or help others employ those markers involved in a syntenic relationship with clinically significant loci for prenatal detection of affected fetuses.

C. METHODS AND PROCEDURE

1. AF Samples

AF samples for these studies are being obtained from appropriate patients by staff physicians with full-time or clinical faculty appointments to the Department of Obstetrics and Gynecology. These physicians provide 10-15 AF samples each year for prenatal diagnosis of chromosomal disorders (primarily trisomy 21); these specimens are usually obtained at 12-16 gestational weeks. We expect the numbers of AF samples sent for prenatal diagnosis will gradually increase as more obstetricians take advantage of the service provided by the Cytogenetics Laboratory at Stanford. One problem in this source of AF samples which concerns us pertains to maternal age. Most of the samples obtained for prenatal diagnosis are taken from women over 35 years of age. The effect of maternal age on expression of polymorphic markers in AF is a variable about which there is no information.

The obstetricians have been cooperative in providing us with AF samples collected at the time of therapeutic abortion; these specimens are usually obtained at 18-20 weeks of gestation. About 2-3 therapeutic abortions are performed each week at Stanford University Hospital.

2. Cultivation of AF CELLS

We shall work with cultivated AF cells in order to assure ourselves that we are working with only fetal cells. After amniocentesis AF usually contains a mixture of maternal blood cells (probably resulting from the procedure) and fetal cells. Although it is possible to remove most of the maternal erythrocytes by lysis (18), other nucleated cells from maternal blood may remain. Cultivation results in dilution of maternal blood cells by the replicating fetal cells.

The use of polymorphic markers and of markers of fetal sex will enable us to decide on the origin of the cells growing in culture (fetal or maternal). Obviously cultured cells carrying a fluorescent interphase Y marker (19) or showing no sex chromatin (Barr) bodies are of fetal origin. For cells which do show evidence of the XX karyotype, we will compare the phenotypes of the polymorphic markers we employ with the phenotypes in the mother and father. In most instances these precautionary procedures will help us avoid mistaking maternal for fetal cells in AF.

We have been cultivating AF cells for the past year. Our culture technique is similar to that used by other investigators in the field. Usually 10 ml of AF (more, if obtained before instilling a hypertonic saline solution for therapeutic abortion) are centrifuged at 100 g for 5-10 minutes. The supernatant AF is removed and saved (see below) and the sedimented cells are suspended in 0.5-1.0 ml of fetal calf serum. We carefully place drops of the serum-cell suspension on the surface of a small plastic Petri dish or on a cover slip in a Petri dish and then incubate the cells at 37 degrees C in a 5% CO2 and air environment. After 6-18 hours, during which time cells have attached to the surface of the dish (or coverslip), we add tissue culture medium (F10-Grand Island Biological Co.) with 30% fetal calf serum. This medium is changed every other day. With this procedure we can see cell growth within one week. There are sufficient cells in a dish or on a coverslip for the first subculture (0.05% trypsinsolution) within 2-3 weeks. After the first subculture, we find that we can perform the second subculture within a week. At this time the cultivated AF cells are growing well.

The cells with which we are dealing at this time usually are epithelioid cells. It is our experience that these cultivated AF epithelioid cells will grow well for about 4-5 subculture passages after which their growth will cease. Within these limits we have been able to cultivate large quantities of these epithelioid cells in roller culture bottles. This will be important for the study of polymorphic enzyme markers in cultivated AF cells.

Less frequently we have noted fibroblasts growing in cultures. These cells grow for longer periods than the epithelioid cells. For instance, one AF fibroblast culture which we are propagating and using for various investigations (7B2B) is now in its 14th subculture passage. We know this line is of fetal origin because it does not show Barr bodies and possesses the XY karyotype; this is a diploid culture. It is our impression that more time is required for fibroblastic growth to be evident initially than for epithelioid cell replication. We will use either morphological type for our studies of cultured AF cells, although for purposes of prenatal diagnosis epithelioid cells may be more relevant.

Aliquots of replicating cells from each AF culture are frozen in 10% DMSO (10**6 cells per ml per vial) and stored in liquid nitrogen for future studies with live cells. Aliquots of 10**7 cells which have been washed, sedimented and drained of supernatant liquid are stored in liquid nitrogen for future electrophoretic and enzyme activity studies.

3. The Supernatant AF Fluid

After AF cells are separated from the supernatant AF by centrifugation, we again centrifuge the latter (2,900 r.p.m. for 10 minutes in an angle head International Clinical Centrifuge) to

remove any remaining cells or large pieces of cell debris. The supernatant AF is then transferred to another container and stored at -20 degrees C. This fraction of AF is used for ABH hemagglutination inhibition studies and will be used for studies on fetal Lewis substances (6).

4. Controls for Expression of Polymorphic Markers

Whenever possible blood samples will be obtained on both parents of the fetus under study. These samples will be typed for the various polymorphic markers being studied in AF. In addition umbilical cord blood specimens will be collected at birth of those fetuses whose AF was studied and who were not aborted. These specimens will also be typed for the various polymorphisms which were studied in the AF. In those instances in which fetuses are aborted, we shall attempt to use appropriate material from the abortuses to type for polymorphisms. The collections of the various specimens just listed are meant to provide us with material which will serve as controls for the observations we make on AF. Working with polymorphic systems permits us to predict the phenotype of the fetus, provided we know maternal and biological paternal phenotypes. A high frequency of discordance between observed (from AF) and expected phenotypes will signal us to examine our testing procedures, to consider that changes are occurring in cell culture or that interesting variations in interallelic expression are occurring. The direct controls of studies of expression of polymorphisms in AF will be the results of testing cord bloods or aborted material.

5. The ABO Secretor and Lewis Polymorphisms

Fetuses which are heterozygous or homozygous for the gene which determines secretion of ABH substances (Se) secrete these soluble blood group antigens which appear in AF (5,6). The secretor type of the fetus and the ABO type of secretor + fetuses can be detected by studying the AF in early gestation free of cells, for hemagglutination inhibition of appropriate detector systems. Inhibition by AF of A, B, or H hemagglutinins indicates that one (or two) of these blood group substances have been secreted by the fetus. In this case the fetus is secretor +, and his ABO type is determined directly by specific inhibition of the A,B or H hemagglutinin. If the AF does not inhibit agglutination the fetus is a non-secretor and the ABO type cannot be determined. About 25% of Caucasian fetuses will be non-secretors (20).

We are already determining in our laboratory secretor status and ABO types of ABH secretor + fetuses. AF is serially diluted for nine or ten doubling dilutions in three series of tubes. In one series of tubes the AF dilutions (including undiluted AF) are incubated at room temperatures for 1 hour with an equal volume of single donor, non-commercial, anti-A hemagglutinin. In the second series of tubes the AF dilutions are incubated with single donor, non-commercial, anti-B and in the third with anti-H (Ulex europaeus). A single dilution of each hemagglutinin is used

throughout the determination, this being determined from the anti-A or anti-B titer of the serum; we do not dilute the anti-H reagent which has a very low titer (commercial preparations or our own preparation). After the incubation of AF and hemagglutinins, we add A, B and H erythrocytes to the first, second and third series of tubes, respectively. Following thorough mixing of cells and reagents (and AF), the tubes are centrifuged and are then observed for gross hemagglutination. As a control for each AF determination, agglutination inhibition is carried out in a similar manner using salivas containing A, B and H substance.

As of the date of preparation of this application, we have standardized (for our laboratory) the conditions of the hemagglutination inhibition procedure (e.g. titer of hemagglutinins, volumes of reagents, incubation times, centrifugation times and speeds) and tested five amniotic fluids:

WEEK OF GESTATION	FETAL ABO	TYPE SECRETOR	RECIPROCAL HEMAGGLUTINATION INHIBITION TITER IN AF		
			A	B	H
19 weeks	B	+	0	64	0
17 1/2 weeks	?	-	0	0	0
16 weeks	A	+	8	0	0
14 weeks	A	+	16	0	0
16 weeks	O	+	0	0	4

We believe that we will be able to distinguish between A1 and A2 secretor fetuses on the basis of inhibition of anti-H; A2 individuals secrete more H substance than do A1 individuals (20). We are continuing to type AF specimens for ABH hemagglutination inhibition and to compare the inhibition titer with that of the saliva controls. We plan to check the ABO types of secretor + fetuses by typing cord blood specimens and ABH secretor status with saliva collected in the newborn period.

In a manner analogous to the methodology just described, secretion of Lewis a, Le(a), and Lewis b, Le(b), substances will be studied (6). Non-secretor fetuses do secrete Le(a) substance.

We are planning to decrease the amount of serological reagents and AF used in the ABH and Le typing procedures. We will attempt to adapt these hemagglutination inhibition tests to tissue typing plates (21) which require only 1 microliter each of AF dilution, antiserum and erythrocytes. These plates can be "centrifuged" on a serological rotator and hemagglutination observed microscopically. Direct reacting (complete) antibodies must be used in this microtechnique. Fortunately, ABH hemagglutinins and some antibodies to Le(a) and Le(b) do not require anti-human globulin (Coomb's reagent) to agglutinate erythrocytes.

6. Other Polymorphic Systems of Erythrocyte Antigens

Recently, the expression of the blood group P has been detected on cultured cells and interspecific cell hybrids by complement fixation (22). If this finding can be confirmed, a technique might be available for detecting the expression of various blood group polymorphisms on cultured AF cells. ABO, Rh, MNS, Duffy, P and Xg specificities will be sought on cultured AF cells by complement fixation. Other serological methods which we will explore for the detection of these substances on AF cells include absorption of specific antisera and mixed agglutination. Fuchs et al. (23) used the latter technique to detect ABO types of uncultured AF cells.

Although there is no indication whatsoever of secretion of blood group antigens other than ABH and Le substances, (except for Sd(a), see reference 20), we shall examine AF for hemagglutinin inhibition activity for the above mentioned erythrocyte antigens.

7. The HL-A System

We have been able to type for HL-A antigens cells in the fifth passage of the fibroblastic euploid culture (7B2B) derived from AF obtained at 19 1/2 weeks of gestation. Cultured AF cells grown to confluence in a plastic tissue culture flask (75 square centimeter growing area) were released from the surface of the flask with 3 ml of 0.05% trypsin solution. The cells were exposed to trypsin for approximately five minutes. They were washed and resuspended in medium F10 without fetal calf serum; the cell concentration of this suspension was 10**6 cells per ml. These cells were prepared and typed for HL-A antigens in Dr. Rose Payne's laboratory, Department of Medicine, Stanford, as follows: Aliquots of the cell suspension were incubated with fluorescein diacetate (FDA 2 micrograms in 0.1 ml tissue culture medium added to each 1 ml aliquot of cell suspension) for 30 minutes and the cells were separated from the FDA by centrifugation. Examination of the FDA-treated, cultured AF cells under the fluorescence microscope revealed masses of fluorescent cells. Aliquots of 1,000-2,000 of these cells were then added to approximately 60 HL-A antisera, representing 25 antigenic specificities, on tissue plates and incubated in the dark at room temperature for 30 minutes. Rabbit serum, absorbed with cultured human fibroblasts, was used as a source of complement; an excess of complement was added to each well of the tissue typing plate containing cells and antibodies. Following an incubation of 1-4 hours at room temperature in the dark, the cells were observed with a fluorescence microscope for evidence of cytotoxicity. Cells which remained fluorescent failed to react with antibodies and thus did not carry the HL-A antigen which the antibodies detect. Fewer or absent fluorescent cells indicated cytotoxicity mediated by the antigens recognized by the antibodies with which the cells had been mixed. Cytotoxicity was graded as 4+ when no fluorescent cells were seen, 3+ few fluorescent cells, 2+ more fluorescent cells and 1+ no decrease in fluorescent cells. This method has been used by Dr. Payne and her associates to detect HL-A antigens on cultured cells (24).

Typing of the 7B2B cells by this method revealed HL-A first series antigens, HL-A9 and W29, and the second series antigen HL-A7. We are especially confident of the detection of HL-A9; most of the anti HL-A9 reagents in Dr. Payne's sublist of antisera reacted with these cells. Although more than one of the antisera for W29 and for HL-A7 reacted with the 7B2B cells, there were some which did not. We are about to type this AF cell culture again, now at passage 14. We are especially interested in the gain or loss of HL-A specificities, especially involving W29 and HL-A7. The genetic structure of the HL-A system (2 closely linked loci with a low frequency of recombination between them, reference 25) serves as a control for our typing results. If we are correctly detecting the HL-A phenotype of the fetus, we should find no more than two first series and two second series antigens on the cultured AF cells. Unfortunately, this fetus was aborted (by hypertonic saline injection), so that we cannot compare the HL-A antigenic type of the cultured AF cells with that of (aborted) fetal tissue or of lymphocytes in cord blood.

We look forward to studying variation in expression of HL-A antigens in cultured AF cells. The genetic structure of the system will permit us to look for variation within sub-series as well as between series (i.e. do the antigens of one sub-series tend to be expressed more frequently in cultured AF cells?) Furthermore, we will study the evolution of expression of HL-A antigens on AF cells throughout the life of the cell culture as well as at different weeks of gestation. Finally, shifts in expression of cross-reacting antigens (26) will be sought.

8. Polymorphic Enzyme Markers of Cultured AF Cells

The demonstration of the electrophoretic phenotypes of G-6-PD and 6-PGD in cultured AF cells by Nadler (7) raises the possibility of detecting other polymorphic enzyme markers. It is not clear from Nadler's report that he considered polymorphism for G-6-PD as the source of the variation he observed in the cultured cells from different AF samples. Nor is it clear that he studied sufficient samples to note variation for 6-PGD in cultured AF cells which he reported as failing to show variation. Three to eight percent of Caucasian individuals show variant 6-PGD electrophoretic phenotypes (27). Harris and Hopkinson (28) have listed some 20 loci determining enzyme structure which have been found by electrophoretic studies to be polymorphic in Europeans, and G-6-PD can be added to this list for Black populations. Of these enzyme polymorphisms we can test extracts of cultured amniotic fluid cells for seven by starch gel electrophoresis: phosphoglucomutase locus 1 (PGM1), phosphoglucomutase locus 3 (PGM3), adenylate kinase (AK), adenosine deaminase (ADA), 6-PGD, hexokinase and G-6-PD. Furthermore, we believe we will be able to develop procedures for five additional systems: Peptidase A, C and D, glutamate-pyruvate transaminase (soluble - SGPT) and galactose-1-phosphate uridyl transferase (gal-1-P-tfase).

Cultured AF cells will be tested for total enzyme activity (per unit weight of cell protein) and for the electrophoretic phenotypes of the systems listed above (many of the procedures which we will use are provided in references in 28). Quantitative determinations and qualitative observations will be made throughout the lifetime of the same AF cell cultures and on cultivated cells from AF specimens taken at different times in early gestation. We will attempt to ascertain all common phenotypes of each polymorphism. Over a three year period we will probably have had the opportunity to encounter the expressions of common alleles for most if not all of these phenotypes.

We shall use standard cell cultures with reference phenotypes to control our electrophoretic procedures. These cultures will be obtained from cell culture repositories (e.g. The Mammalian Genetic Mutant Cell Repository, Institute for Medical Research) or from biopsies (after informed consent procedures) from individuals who have been typed for various enzyme electrophoretic phenotypes.

9. Applications to Prenatal Diagnosis

As this study progresses we will become familiar with the behavior and the limits of variation of expression of various polymorphic markers in AF. This information will serve as a base for using syntenic relationships for prenatal diagnosis. We envision a cooperative effort with a number of other centers involved in genetic counseling and prenatal diagnostic activities in finding families who are at risk for having affected fetuses and to which the methodology discussed herein can be applied for prenatal diagnosis of those fetuses. Such families will be typed for the test marker in order to determine that one or both (for autosomal recessive traits) parents are heterozygous and to work out the coupling phase. We will then seek the expression of the syntenic marker in the AF from those informative and suitable pregnancies. Certainly, families with hereditary disorders seen at Stanford will be screened for the possibility of applying the methodology presented in this application, but we believe we will need access to a larger number of families to apply this prenatal diagnostic technique.

D. SIGNIFICANCE

1. This project will enable us to infer information about well known polymorphic markers in the developing fetus. This includes the development of the expression of these markers in early antenatal life. The project gives us the opportunity to find fetal forms of these markers. By understanding differential expression of polymorphic alleles in fetal life, we may be able to make inferences about the nature of selective forces which may be acting on these polymorphisms.

2. This project could ultimately result in expanding the number of hereditary conditions amenable to prenatal diagnostic techniques. This is especially true for autosomal dominant

disorders for which little information on basic biochemical mechanisms is available to fashion appropriate techniques for prenatal detection of the fetal phenotype.

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1. PERSONNEL (List all personnel engaged on project) NAME (Last, first, initial)	TITLE OF POSITION	TIME OR EFFORT %/HRS.	AMOUNT REQUESTED (Omit cents) TOTAL
Cann, H.	Principal Investigator or Program Director	20%	POLYMORPHIC GENETIC MARKERS
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Open - Pediatrics	Research Assistant	50%	
Cusumano, M.	Laboratory Diener	50%	
Murray, R.	Secretary	25%	

TOTAL →	$ 52,330
2. CONSULTANT COSTS (Include Fees and Travel)	$
3. EQUIPMENT (Itemize) Eppendorf Microfuge and accessories $500 Buchler Power Supply (constant voltage/current) $600	$ 1,100 *
4. SUPPLIES Chemicals, antisera, tissue culture supplies, glassware, etc.	$ 5,000
5. STAFF TRAVEL (See Instructions) a. DOMESTIC 1 East Coast Meeting	$ 500
5. STAFF TRAVEL (See Instructions) b. FOREIGN	$
6. PATIENT COSTS (Separate Inpatient and Outpatient)	$
7. ALTERATIONS AND RENOVATIONS	$
8. OTHER EXPENSES (Itemize per instructions) Office supplies, telephone, repro., postage, publication costs, etc. $400 Central computer usage and terminal rental $1600	$ 2,000
9. Subtotal – Items 1 thru 8 →	$ 60,930

FOR TRAINING GRANTS ONLY

10. TRAINEE EXPENSES (See Instructions)			
a. STIPENDS	PREDOCTORAL	No. Proposed ____	$
	POSTDOCTORAL	No. Proposed ____	$
	OTHER (Specify)	No. Proposed ____	$
	DEPENDENCY ALLOWANCE		$
	TOTAL STIPEND EXPENSES →		$
b. TUITION AND FEES			$
c. TRAINEE TRAVEL (Describe)			$
11. Subtotal – Trainee Expenses →			$

12. TOTAL DIRECT COST (Add Subtotals, Items 9 and 11, and enter on Page 1) →	$ 60,930

POLYMORPHIC GENETIC MARKERS

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)

DESCRIPTION	1ST PERIOD (SAME AS DETAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED *(This application only)*					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	52,330	56,035	59,897	64,022	68,424		
CONSULTANT COSTS *(Include fees, travel, etc.)*							
EQUIPMENT	1,100*						
SUPPLIES	5,000	5,300	5,700	6,000	6,400		
TRAVEL – DOMESTIC	500	600	600	600	700		
TRAVEL – FOREIGN							
PATIENT COSTS							
ALTERATIONS AND RENOVATIONS							
OTHER EXPENSES	2,000	2,100	2,300	2,500	2,600		
TOTAL DIRECT COSTS	60,930	64,035	68,497	73,122	78,124		

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD *(Enter on Page 1, Item 4)* → $ 344,708

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Budget explanation attached.

BUDGET EXPLANATION

Professor Cann's and Dr. Tsuboi's time are each budgetted at 20% in support of this project. The project also requires two full time and one part time research assistants. The part time research assistant will assist in typing cultured cells from amniotic fluid for HL-A antigens. One full time assistant (Mrs Van West) will assist in the development of serologic tests on amniotic fluid and amniotic fluid cells. The other research assistant (G. Makk) will assist in culturing amniotic fluid cells and in performing electrophoresis on cultured cell extracts. The laboratory diener will provide the needed support for cell culture work (washing, sterilization of equipment, etc) and also for general laboratory work. We are also requesting 25% of secretarial time to support the activities of both professionals in this project.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

The microfuge will be used to centrifuge small amounts of cultured cells. The power pack will be used for electrophoresis. Necessary supplies are budgetted to support laboratory work including chemicals, glassware, antisera, etc.

We are requesting $100 per month for computer time and data file storage and $400 per year for our share of the rental of a computer terminal. Access to computation is necessary for cataloging of cultured cells from AF specimens, automatic filing and storage of test results on various cell cultures and amniotic fluid samples, filing and storage of data on phenotypes of parents, relating parental data to fetal data, monitoring lists of expected dates of delivery of fetuses we have studied and data analysis.

SECTION V

A Search for Genetic Polymorphisms and Variances Of Specific Binding Proteins in Blood

Dr. Cavalli-Sforza

Genetic Polymorphisms and Variants of Specific Binding Proteins in Blood

Dr. L. L. Cavalli-Sforza, Principal Investigator

A. INTRODUCTION

A.1 Objective

To search for new medically significant genetic variants and genetic polymorphisms of specific binding proteins in blood.

A.2 Background and Rationale

The recognition of genetic differences by electrophoretic studies of proteins is by now a widely applied procedure. Methods employed for detecting proteins after electrophoresis range from unspecific protein staining to the identification of specific proteins by a great variety of techniques. Harris and Hopkinson (1972) have recently summarized the evidence collected in electrophoretic studies of specific enzymes in Caucasians. Out of 7 enzymes analyzed, about 1/3 were found to be polymorphic. Certain other categories of enzymes may be subject to a lower rate of polymorphism (Omenn, Cohen and Motulsky, 1971).

One method of labeling a variety of proteins is already established but has not been tested systematically for its capacity to identify polymorphisms or rare variants. It consists of adding to serum (or other biological fluids) a radioactive ligand molecule and following its binding to a specific protein (or proteins) by electrophoresis followed by autoradiography. This method was used (Giblett, Hickman and Smithies, 1959) to amplify the classical notion that transferrin binds iron and that electrophoretic variants of this molecule retain the capacity to bind iron. The physiological function of this protein is fairly well known and its important role in the organism well ascertained (for details see Giblett, 1969). Polymorphism is well documented and many rare variants are also known. Other proteins known to specifically bind metals (e.g. copper: ceruloplasmin, see Giblett for details) and other substances (e.g. thyroxine; see Heinonen et al., Fialkow et al., and Penfold et al.) have also been studied.

The suggestion advanced in the present application is to test systematically samples of human blood for proteins binding specific substances, taking advantage of the fact that many physiologically important ligands are available in a radioactive form. Two aims could be thus obtained: 1) increase the present wealth of polymorphisms, a very desirable aim (see Cavalli-Sforza, 1973); 2) a functional basis for each specific polymorphism or rare variant could be sought, given that the nature of the substance would usually suggest possible advantages or handicaps of the variants.

B. SPECIFIC AIMS

(1) Search for polymorphisms in proteins binding specific substances available in radioactive form. This may enable us to look for a large number of new polymorphisms, allowing us to considerably increase the genetic map and to find new linkages.

(2) In all cases in which polymorphisms are detected, test for developmental behavior of the proteins in question, and for the chemical specificity of the ligand.

(3) In cases of toxicity or idiosyncratic drug reaction, test for variation or absence of proteins binding the specific poison or drug.

C. METHODS AND PROCEDURE

The experimental methodology has already been developed on the basis of experience accumulated in another research line. In the analysis of platelet proteins binding certain neurotransmitters (in particular, serotonin and norepinephrine) a method of electrophoresis followed by autoradiography was set up. In order to test the method for its capacity to reveal serum proteins binding specific substances, it was applied to lead-binding proteins. A short description of the method follows: Pb(210) at an appropriate concentration is incubated at 37 degrees C. for 30 minutes with human serum. 0.1 ml of the incubated serum is electrophoresed on acrylamide gel. Bromophenol blue is used as a tracking dye and electrophoresis stopped when the dye is at the bottom of the column. (The procedure can be used both on columns and on slab gels). At the end of electrophoresis, the gel column is cut longitudinally and the flat surface applied on an X-ray Kodak film for 4-10 days in conditions guaranteeing close adhesion and maximum efficiency of arrival to the plate of the electrons emitted by the decaying radioactive atoms. A diagram of a developed autoradiogram is shown in Figure 1.

Four different individuals are shown. There is considerable binding of Pb to albumin which forms the large thick band. Albumin is known to have a high affinity for many substances. In addition, there are other lighter bands of Pb-binding proteins in the globulin region. Some of these correspond to thin protein bands visible by staining. Two proteins in the beta globulin region have been constantly found in all individuals tested so far. In the gel at the extreme left, two bands are visible near the origin (the top of the figure) while there is only one band at the same location in the other three individuals. The double band may be due to heterozygosis but so far only one individual with a double band has been found on the limited number of individuals tested. It is planned to continue the testing on at least a hundred normal individuals, to establish if a polymorphism really exists, to test the families of individuals showing electrophoretic differences, and to test all cases of plumbism which may come to our attention by the cooperation of

the hospital staff. Some of these individuals may be idiosyncratically sensitive to lead; or, alternatively, may show physiological responses to lead intoxication. Other disease states suspected of affecting plasma protein distributions will also be examined.

The extension of this work to substances other than lead is the subject of the present grant application. The substances to be tested for binding to serum proteins can be many, the main limitation being that the organic substances must be labelled with C(14) or otherwise produce beta radiation of similar energy. Pb emits also gamma-radiation, so that exposed plates must be shielded; this, however, is no serious limitation since contact of the experimenter with the radioactive material is very short and his or her exposure controlled by the supervision ordinarily carried out in the laboratory (film badges, routine checks of benches and equipment). Results with H(3) labelled material have so far been unsatisfactory due to the short range of the weak electrons emitted in H(3) decay. C(14) labelled material is entirely satisfactory, as shown by the previous experience with platelets.

A shortened list of the substances to be tested includes the following:

(1) Elements which have radioactive isotopes suitable for the test.

(2) Amino acids, peptides

(3) Nucleotides, nucleosides

(4) Sugars

(5) Lipids

(6) Hormones

(7) Vitamins

(8) Drugs, including antibiotics, addiction drugs, pesticides, poisons, etc.

The analysis should be carried out initially on a sample of at least one hundred random individuals. Over 100 substances can be tested, but each test requires only 0.1 ml of serum and thus 20-30 ml of blood obtained from an individual will be enough for all tests. On the assumption that one or more proteins binding specifically a substance are found for most of these substances, this experiment should give a good sample of prospective polymorphisms from which the frequency of polymorphism and average heterozygosity for each can be computed and compared with that observed for enzymes (see Introduction).

Substances found to have polymorphic binding proteins can then be subject to the following series of observations:

1) Tests on families scored for other markers which have already been collected in other laboratories. Prospective collaborations are being considered. It is expected (but should be first tested) that in serum stored in freezers the specific binding activity is stable. The existence of a number of projects in which blood samples have been collected from families, examined and stored makes it easier and more efficient to test on such material inheritance of the protein differences (i.e. segregation analysis) and linkage of the corresponding genes to standard markers. Several such collections of samples are already available.

2) We plan to examine newborn infants born at Stanford Hospital of matings in which the mother is homozygous for a polymorphic protein of the type described, and the father heterozygous (or homozygous for another allele). The paternal protein would be searched in cord blood and if not present, the child would be followed further to establish the age of appearance of the paternal protein. This would give us a chance to seek regulatory genes for the developmental pattern of these proteins. For instance, we will seek variation among individuals of age of appearance of the protein and analyze the variation with family studies.

3) For every specific substance, patients with diseases that may be explained by a variation or absence of a binding protein, the specific substance should be examined.

D. SIGNIFICANCE

It is difficult to anticipate the total number of proteins that can be identified by this procedure, but existing information would suggest that it can be as high as several hundred. The method suggested then supplies a very economical procedure for testing a great number of potential polymorphisms. The frequency of polymorphic genes is one of the quantities which is of interest to estimate for comparison with the existing enzyme data. This result has obvious evolutionary significance in view of the present discussion on neutrality of polymorphic genes. If the proportion is the same as is known to be among enzymes, then this investigation may generate enough markers to more than double the existing genetic map of man, with all consequent advantages of increased precision in genetic counseling and research.

The interest offered by such new polymorphisms would be greatly enhanced by the possibility of detecting variation for regulatory genes in the manner explained before. This is one of the most difficult fields in human general genetics today, the development of which may be most fruitful.